



Original Article

Moesin overexpression is a unique biomarker of adenomyosis

Rena Ohara,^{1,2*} Hiroo Michikami,^{1,2*} Yuko Nakamura,^{1,2} Akiko Sakata,¹ Shingo Sakashita,¹ Kaishi Satomi,¹ Aya Shiba-Ishii,¹ Junko Kano,¹ Hiroyuki Yoshikawa² and Masayuki Noguchi¹

Departments of ¹Pathology and ²Obstetrics and Gynecology, Faculty of Medicine, University of Tsukuba, Tsukuba-shi, Japan

Adenomyosis is characterized by extension of endometrial glands and stromal cells into the myometrium. Here we proved that ‘moesin’ is a unique biomarker of adenomyosis. We selected two cases of adenomyosis that had been surgically resected and fixed with formalin. Proteins were extracted from the infiltrating adenomyosis lesions and normal endometrium by tissue microdissection. The extracted proteins were examined using a LC-MS/MS system and the expression profiles of each region were compared. Two hundred and sixty proteins were detected, among which 73 were expressed more in adenomyosis than in normal endometrium. Among these proteins, we focused on overexpression of moesin in adenomyosis. Expression of moesin estimated semiquantitatively using an immunohistochemistry score was higher in adenomyosis than in normal endometrium. In particular, moesin was significantly overexpressed in stromal cells of adenomyosis than in those of normal endometrium. Relative to normal endometrium, moesin was also overexpressed at the RNA level in 9 of 14 cases of adenomyosis and at the protein level in all 14 cases. We also detected activated (phosphorylated) moesin in adenomyosis lesions. The present findings suggest that moesin is characteristically overexpressed and activated in adenomyosis, and that moesin activation may be related to extension of adenomyosis in the myometrium.

Key words: adenomyosis, invasion, LC-MS/MS analysis, moesin

Adenomyosis is an estrogen-dependent disease that affects 8–62% of women of reproductive age, being defined as the presence of ectopic endometrial glands and stromal cells within the myometrium.^{1,2} Although adenomyosis is a benign gynecological disease, it shows characteristic ‘amoeba-like’ invasive growth of both the endometrial columnar epithelium and stromal cells; sometimes only stromal cells may be pathologically evident. However, the molecular mechanism responsible for its pathogenesis has not yet been determined. Three main theories to explain the development of adenomyosis have been proposed: the direct invasion theory, the mullerian residual theory, and the transplantation theory. The most well known of these theories contends that adenomyosis is the result of downward extension of the endometrium from the uterine cavity due to collapse of the endometrial basal layer, and that increased invasiveness of the endometrial cells may be responsible for the development of adenomyosis.³ Recently, some articles have indicated that the epithelial-mesenchymal transition (EMT) is the critical mechanism responsible for development of adenomyosis.⁴ However, the mechanisms of invasion during adenomyosis development remain elusive. Although the invading endometrial tissue has no malignant potential, its pattern of invasive growth bears many of the hallmarks of malignancy.

In the present study, we performed proteomics analysis using a LC-MS/MS system, and searched for molecules overexpressed in adenomyosis. For this purpose, we used formalin-fixed surgical specimens of adenomyosis. Proteins were extracted from both normal endometrium and invasive lesions of adenomyosis that had been separated using a laser-capture microdissection system, and their expression profiles were compared. Molecules that showed significantly higher expression in adenomyosis than in normal endometrium were selected. This approach led to the recognition of moesin as a unique biomarker of adenomyosis.

Correspondence: Masayuki Noguchi, MD, Department of Pathology, Faculty of Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba-shi, Ibaraki 305-8575, Japan. Email: nmasayuk@md.tsukuba.ac.jp

*Authors RO and HM contributed equally to this paper.

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MATERIALS AND METHODS

Patients

We selected 37 women with adenomyosis who underwent transabdominal hysterectomy at Tsukuba University Hospital (Ibaraki, Japan) between 1998 and 2012. The patients included those who had been diagnosed as having adenomyosis both before and after surgery. All of the surgical specimens were formalin-fixed and paraffin-embedded. Among these cases, two were used for LC-MS/MS analysis and the other 35 were used for immunohistochemistry. In addition, 14 fresh specimens surgically resected at Kasumigaura Medical Center (Ibaraki, Japan) in 2011 were used for real-time RT-PCR and Western blotting. Furthermore, two sets of adenomyosis specimens were also examined for moesin phosphorylation using a phosphorylated specific anti-moesin antibody. The ethics committees of the two hospitals approved this study, and informed consent for specimen collection was obtained from all of the patients concerned.

Laser microdissection and LC-MS/MS analysis

We performed laser microdissection using a Leica CTR6000 (Leica Microsystems, Wetzlar, Germany) and collected protein from adenomyosis lesions and normal endometrial tissue individually. The collected proteins were trypsinized using a Liquid Tissue MS Protein Prep kit (Expression Pathology, Gaithersburg, MD, USA). Peptide samples were separated by liquid chromatography and analysis of peptide masses was performed using mass spectrometry (ZAPLOUS LC/MS Systems, AMR, Tokyo, Japan). A database search was performed using the MASCOT search engine (Matrix Science, London, UK) in the IPI human database.

Antibodies and reagents

A rabbit anti-moesin antibody and a mouse anti- β -actin antibody (Sigma, Bornem, Belgium) were also used for Western blot analysis. Anti-lumican antibody raised in goat (R&D Systems, Minneapolis, MN, USA), anti-moesin antibody raised in rabbit (Sigma), and anti-triosephosphate isomerase antibody raised in rabbit (Novus Biological, Littleton, CO, USA) were used for immunohistochemistry. A Histofine simple stain MAX-PO goat (Nichirei, Tokyo, Japan) and an Envision kit HRP (Dako, Glostrup, Denmark) were used as the secondary antibody. An anti-p-moesin antibody raised in rabbit (T558 phosphorylation, Santa Cruz, Santa Cruz, CA, USA) was obtained for phosphorylation analysis and used for Western blotting.

Immunohistochemistry

After deparaffinization, the samples were placed in blocking solution to suppress non-specific staining for 30 min. Then, epitope retrieval was performed using citrate buffer (pH 6.0) in an autoclave at 121°C for 10 min. Tissue slices were incubated with the antibody in accordance with our laboratory manual. For each case, a semi-quantitative moesin expression score was calculated based on the percentage of positive epithelial and stromal cells and their immunostaining intensity in adenomyosis and normal endometrium. The immunostaining intensity was scored as: 1, negative or trace; 2, weak; 3, moderate; or 4, intense. The total score was calculated by multiplying the intensity score and the fraction score, to give a score range of 100–400.

Collection of total RNAs and real-time RT-PCR

Nine 20- μ m sections of fresh frozen tissue were placed on laser microdissection slides (DIRECTOR, Expression Pathology), then fixed with 5% (v/v) acetic acid/ethanol and stained with 0.05% toluidine blue prepared in our laboratory. Using a laser microdissection system, samples were collected from adenomyosis lesions and normal endometrial tissue individually. Total RNAs were extracted using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

Real-time RT-PCR was performed using SYBR Premix Ex Taq™ II (TaKaRa, Otsu, Japan) and 7300 Real-Time PCR (Applied Biosystems) systems. The template was 1.5 ng of cDNA in 80 μ l of PCR mixture containing 6.4 μ l of 5 μ M moesin forward primer and 5 μ M moesin reverse primer, and dispensed in 25- μ l aliquots. The same amount of β -actin was used as an internal control. The sequences of the moesin primers were '5-AGCCTGTGCCCTGATCCAAG-3' (forward) and 5'-GGGACAGAACAGCTGGTGTCAA-3' (reverse).

Western blot analysis

Twenty 10- μ m cryostat sections of adenomyosis and endometrium were prepared from each frozen specimen. The sections were collected with 500 μ l of T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL, USA) and 5 μ l of Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Waltham, MA, USA). Amicon Ultra-0.5 3 K (Millipore, Billerica, MA, USA) was used to concentrate the extracted protein in accordance with the manufacturer's instructions. Twenty micrograms of extracted protein was prepared for samples, subjected to electrophoresis on 12% polyacrylamide gel (SDS-PAGE), and transferred to

polyvinylidene difluoride (PVDF) membranes using an iBlot gel transfer system (Invitrogen, Carlsbad, CA, USA). The membranes were blocked with 0.1% blocking reagent and then incubated with the primary antibody. Protein bands were visualized using Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

Statistical analysis

Data was analyzed using JMP 9 (SAS Institute Inc., Cary, NC, USA). Statistical significance of differences was evaluated by Pearson's chi-squared test, Student's *t* test, Wilcoxon matched pairs signed rank test or linear regression analysis. Differences at $P < 0.05$ were considered significant.

RESULTS

We extracted the proteins from both normal endometrium and invasive areas of two adenomyosis cases using a tissue microdissection system and performed a proteomics study using LC-MS/MS. We screened proteins that showed higher expression in adenomyosis than in normal endometrium in

both cases. In total, 260 proteins were detected, of which 73 showed higher expression in adenomyosis than in normal endometrium on the basis of spectrum counts (Table S1). These 73 proteins included binding proteins, structural proteins, and signal transcription factors. Among them, we focused on lumican, triosephosphate isomerase (TPI), and moesin, whose expression is known to be correlated with the invasive growth of malignant tumors.^{5–11}

The expressions of these three genes were examined by immunohistochemistry using 35 surgically resected specimens of adenomyosis. For epithelial cells, twenty-three (65.7%) of the 35 cases, and for stromal cells, 26 cases (72.3%) showed higher moesin expression in adenomyosis than in normal endometrium (Figs 1,2). Interestingly, moesin expression was detected mainly in stromal cells relative to epithelial cells (Fig. 1d). Although the immunohistochemistry (IHC) score for moesin in adenomyosis (both epithelial cells and stromal cells) was higher than that in normal endometrium (Table 1), overexpression of moesin was significantly stronger in stromal cells of adenomyosis than in those of normal endometrium (Table 1, $P < 0.001$) (Fig. 2). The other two proteins (lumican and TPI) showed no significant difference of staining score between adenomyosis and normal endometrium (data not shown). The high expression of moesin in

Figure 1 Immunohistochemical analysis of moesin in surgical sections of adenomyosis. (a) Macroscopic features evident in a H&E section. The surgical specimen was mainly composed of three histological structures: an adenomyosis area (ADM), a myometrium area (MM), and an endometrium area (EM). (b) Immunohistochemistry for moesin (magnification, x40). (c) 1 (Endometrium) Scattered expression of moesin was observed in endometrial tissue (both epithelial cells and stromal cells) (magnification, x400). Scale bar, 50 μ m. (d) 2 (Adenomyosis) Moesin was overexpressed in the adenomyosis area. Both epithelial cells and stromal cells expressed moesin, but stromal cells showed a higher level of expression than epithelial cells (magnification, x400). Scale bar, 50 μ m

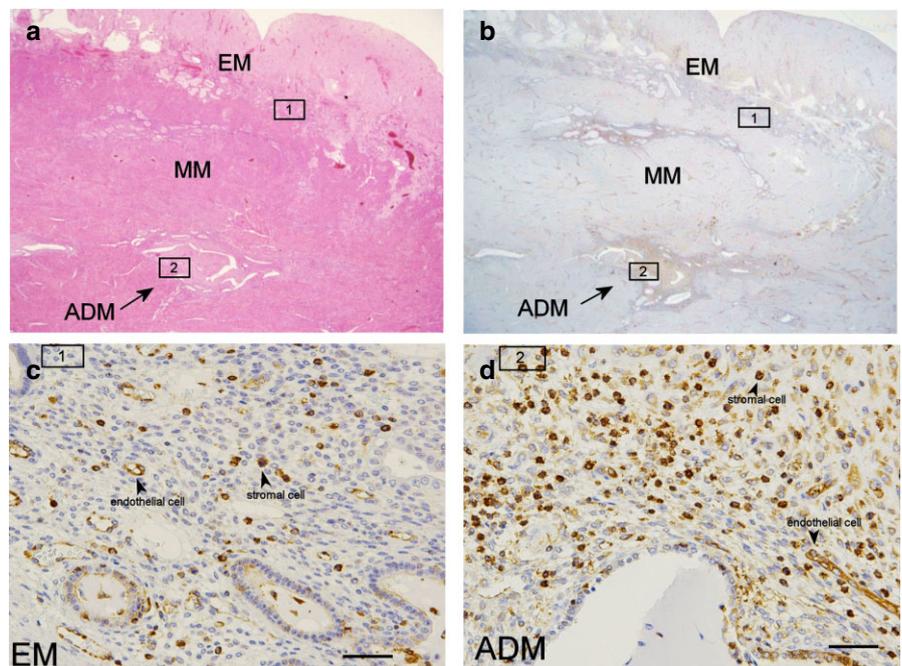


Table 1 Moesin expression in adenomyosis and normal endometrium

	Adenomyosis	Normal endometrium	<i>P</i> value
Epithelial cell IHC score; median (range)	108 (100–176)	103 (100–183)	$P = 0.098$ (Wilcoxon matched pairs signed rank test)
Stromal cell IHC score; median (range)	140 (113–236)	123 (100–200)	$P < 0.001$ (Wilcoxon matched pairs signed rank test)

IHC, immunohistochemistry.

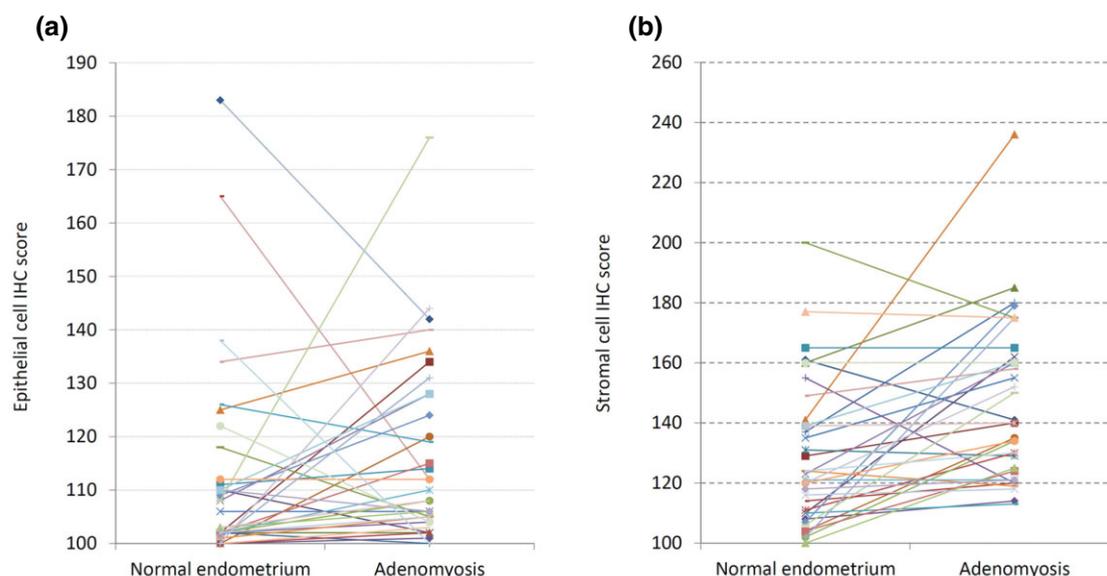


Figure 2 Comparison of the moesin immunohistochemical expression scores between normal endometrium and adenomyosis. (a) epithelial cells, (b) stromal cells. (* $P < 0.05$: Wilcoxon matched pairs signed rank test.)

stromal cells showed no significant relationship with any stage of the menstrual cycle, or with other clinical parameters in the individual patients (Table S2-1), but in epithelial cells, high expression of moesin was significantly associated with a history of uterine surgery and hormone therapy (Table S2-2). We then examined the relationship between depth of invasion and IHC score in both epithelial cells and stromal cells (Fig. 3a,b). The IHC scores for both epithelial cells and stromal cells were inversely correlated with invasion depth.

To confirm the overexpression of moesin in adenomyosis, we performed real-time RT-PCR for moesin using 14 fresh specimens of surgically resected adenomyosis. All of the samples employed were extracted using laser microdissection. Nine (64%) of the 14 cases examined showed higher expression of moesin in adenomyosis than in normal endometrium (Fig. 4).

High expression of moesin was also confirmed by Western blotting using the 14 fresh specimens that had been employed for real-time RT-PCR. In this experiment, we did not subject the tissue samples to microdissection, and the proteins extracted from adenomyosis lesions included those from myometrial tissue. However, unlike the results of real-time RT-PCR, all 14 cases showed higher expression of moesin in adenomyosis than in normal endometrium (Fig. 5). The expression of moesin was evaluated using the Image J software package (National Institutes of Health, Bethesda, MD, USA), and shown to be statistically significant ($P = 0.0006$).

Several articles have reported that activation of moesin requires phosphorylation of T558 in the moesin molecule. Therefore, we examined moesin activation (phosphorylation)

in two specimens of adenomyosis. As shown in Fig. 6, adenomyosis expressed phosphorylated moesin, whereas normal endometrial tissue showed no signal.

DISCUSSION

In this study using LC-MS/MS analysis, we focused on possible biomarkers of adenomyosis by screening proteins that showed higher expression in adenomyosis than in normal counterpart endometrial tissue, and then using immunohistochemistry found that 72.3% (26/35) of adenomyosis cases showed higher expression of moesin than normal endometrium. These results were confirmed at both the RNA (RT-PCR) and protein (Western blotting) levels. Therefore, although the histology of adenomyosis mimics normal endometrial tissue, moesin overexpression is a unique characteristic of adenomyosis.

Moesin overexpression is reportedly related to carcinogenesis in various carcinomas, such as pancreatic cancer¹² or colorectal carcinoma,¹³ and also to the invasiveness of melanoma cells.¹⁰ Kobayashi *et al.* have demonstrated that the expression pattern of moesin is an independent prognostic factor in oral squamous cell carcinoma.¹⁴ On the other hand, Ou-Yang *et al.* have shown that transfection with an ERM-targeted siRNA expression vector reduced the invasive growth of a gastric cancer cell line, SGC-7901.¹⁵ Carmeci *et al.* have also reported that high expression of moesin contributes to the invasiveness of hormone-unresponsive breast cancers.¹⁶ Our data showed that moesin expression was not related to any stage of the menstrual cycle, indicating

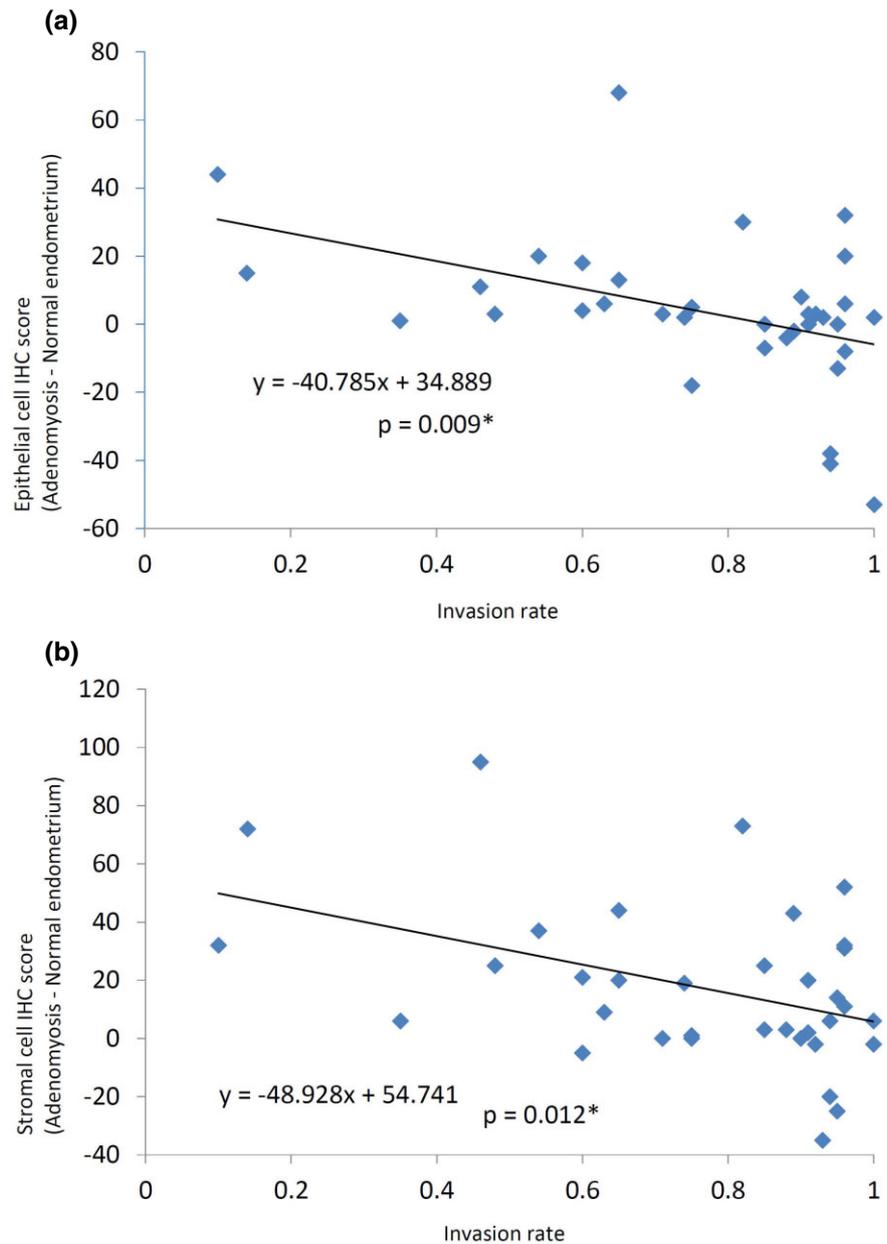


Figure 3 Correlation between immuno-histochemical (IHC) score and degree of invasion (0 to 1). Both **(a)** the epithelial cell IHC score and **(b)** the stromal cell IHC score were inversely correlated with the degree of invasion ($*P < 0.05$: linear regression analysis).

that high expression of moesin is not associated with any hormone-dependent mechanism of invasiveness in adenomyosis.

Moesin is a well conserved gene in many species and is present in many tissues, exerting various molecular actions, such as regulation of the actin cytoskeleton and control of cell shape, cell adhesion or motility.¹⁷ Phosphorylation is required for activation of moesin, and different activation signals regulate the functions of moesin by modulating these intramolecular interactions. Phosphorylation of moesin acts as a switch to trigger cell motility. In this study we demonstrated the presence of phosphorylated moesin in surgically resected specimens of adenomyosis, but not in normal endometrial tissue (Fig. 6). The present results suggest that

overexpression of activated moesin is not only one of the phenotypic characteristics of adenomyosis, but also that cells expressing phosphorylated (activated) moesin in adenomyosis have some functional role in its extension into the myometrium. Interestingly, moesin IHC scores for both epithelial cells and stromal cells were inversely correlated with the depth of adenomyosis invasion (Fig. 3a,b). Therefore, we speculate that activated moesin is critical for early downward extension of adenomyosis. However, the relationship between moesin over-expression and tumor cell invasion is still debatable. In melanoma, it is reported that immunohistochemical expression of moesin decreases according to the extent of invasion.¹⁸ On the other hand, in gastric adenocarcinoma, moesin expression increases according to the extent

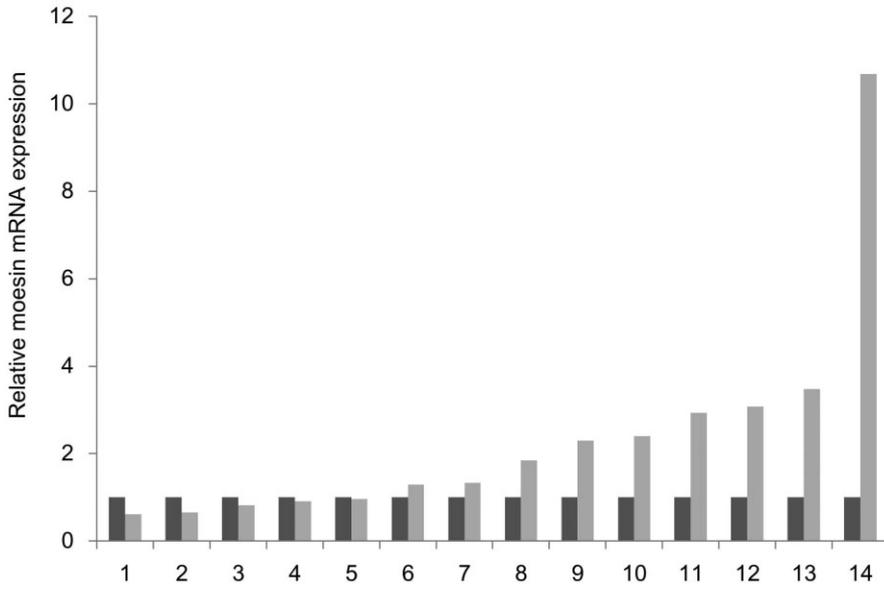


Figure 4 Real-time RT-PCR analysis of moesin mRNA expression in adenomyosis lesions and normal endometrium. Using 14 cases of adenomyosis, the relative expression of moesin was examined by real-time RT-PCR. Relative expression of moesin mRNA in adenomyosis was compared with that in normal endometrium. ■; normal endometrium, ■; adenomyosis lesion (* $P < 0.05$; Wilcoxon matched pairs signed rank test.)

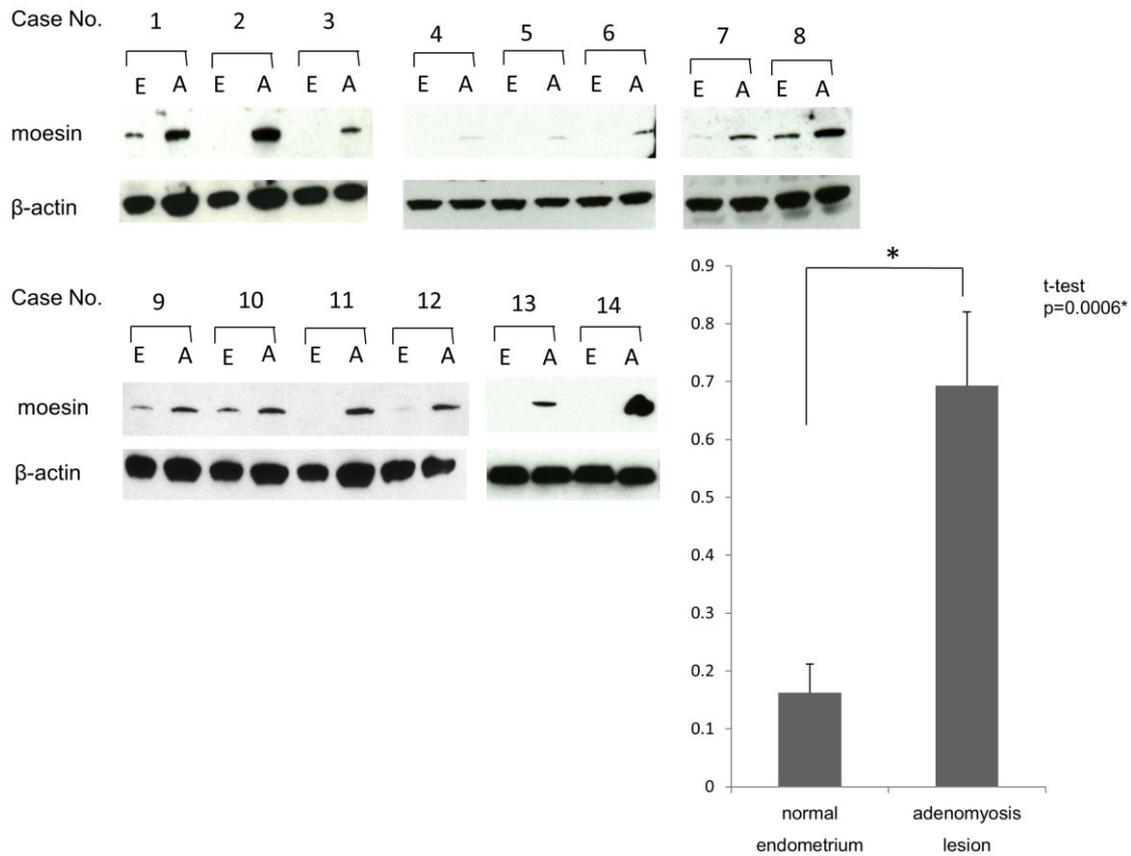


Figure 5 Western blot analysis of moesin expression in surgically resected cases of adenomyosis. Using 14 cases of adenomyosis, the relative expression of moesin was examined by Western blotting (* $P < 0.05$; Student's t test). 'E'; normal endometrium, 'A'; adenomyosis in each case. Internal control was β -actin. (■) moesin expression/ β -actin expression.

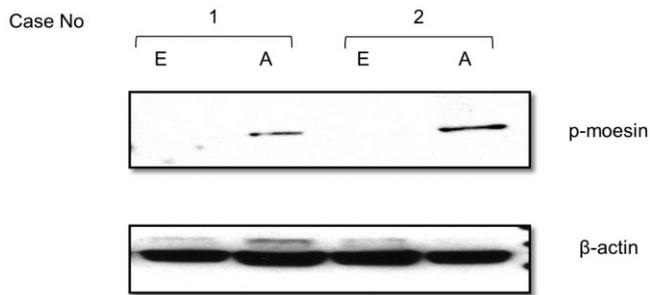


Figure 6 Western blotting of phosphorylated moesin in surgically resected cases of adenomyosis. Phosphorylated moesin was detected in adenomyosis lesions but not in normal endometrium. E, endometrium; A, adenomyosis.

of invasion.¹⁹ Further details of the relationship of moesin to invasiveness await further studies using a specific antibody against phosphorylated moesin. In order to clarify the molecular mechanism responsible for the infiltrative activity of adenomyosis and to screen potentially useful therapeutic drugs, there is a need to examine in detail the mechanism of moesin phosphorylation in adenomyosis.

It was noteworthy that IHC for adenomyosis using an anti-moesin antibody demonstrated greater overexpression of moesin in stromal cells than in epithelial cells (Table 1, Figs 1,2). Recently, moesin has been reported to be an EMT marker or EMT-related gene.^{20–22} For example, Hynes *et al.* demonstrated that increased moesin expression was related to actin remodeling during EMT. Also, Takahashi *et al.* have reported a relationship between moesin and CD44, which is a cancer stem cell marker. Gargett *et al.* reported that stromal clones isolated from endometrium expressed CD44, a mesenchymal stem cell marker.^{23,24} In our present study, we were unable to clarify any association between moesin overexpression in stromal cells of adenomyosis and the EMT, but stromal cells showing high expression of the moesin gene might be associated with the pathogenesis of adenomyosis (mixed proliferation of the epithelial and stromal components).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Seventy-three proteins found to show higher expression in adenomyosis lesions in LC-MS/MS analysis.

Table S2. Patients and clinical characteristics of adenomyosis cases used in this study. Statistical analysis of the relationships between moesin expression and clinical data was performed ($P < 0.05$).